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Ca2+/Calmodulin Binding to PSD-95 Mediates Homeostatic Synaptic Scaling Down

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Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   08 November 2016

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that the overall opinion is not very positive.

The referees appreciate that the analysis adds new insight. However, I am also afraid that they raise significant concerns that also preclude publication here. In particular the referees raise concerns regarding the data supporting that that CaM binding to PSD-95 promotes homeostatic synaptic down scaling. Both referees #1 and 2 find that data supporting this conclusion is not strong enough and find that much further work is needed to demonstrate this. As you probably can discern from the comments, referee #3 was asked to evaluate the NMR data. Given these comments from good experts in the field, I am afraid that I can't offer to invite a revision.

I am very sorry that I can't be more positive on this occasion, but I hope that you will find the referees' comments constructive and useful.

REFEREE REPORTS

Referee #1:

This study by Turner et al. presents some very interesting new findings regarding the mechanisms through which chronic increases in neuronal activity that induce homeostatic synaptic plasticity to globally decrease excitatory synaptic strength (i.e. down-scaling)controls the subcellular

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localizations of the key postsynaptic density (PSD) scaffolding molecule, PSD-95, and associated AMPA-type glutamate receptors (AMPARs) that it regulates. This work builds on previous structural and functional studies from the same group of investigators showing that Ca2+-calmodulin (CaM) binds to the N-terminus of PSD-95 to inhibit its synaptic targeting by preventing palmitoylation/re-palmitoylation of Cys3 and Cys5, modifications which are essential for PSD-95 membrane and PSD targeting (Zhang et al., 2014 EMBO J). Here the authors go on to show that phosphorylation of T19 in this PSD-95 N-terminal domain increases the affinity of Ca2+-CaM binding to PSD-95. This T19-P modification was previously shown by Morgan Sheng and colleagues to be required during the induction of long-term depression (LTD), another form of plasticity that decreases excitatory synaptic strength by promoting PSD-95 and AMPAR removal from synapses (Nelson et al., 2013 J Neurosci).

Importantly,through using NMR to determine the structure of Ca2+-CaM bound to PSD-95pT19, Turner et al. were able to identify novel electrostatic interactions between residues in CaM and PSD-95 that contribute to increased Ca2+-CaM binding affinity compared to non-phosphorylated PSD-95, including interaction of PSD-95 E17 with CaM R126. Charge reversal mutation of these key residues (including E17R and T17L in PSD-95) not only decreased the affinity of Ca2+-CaM binding to PSD-95 N-terminus in vitro but also prevented de-clustering of PSD-95 and reduction in plasma membrane surface expression of the AMPAR GluA1 subunit in response chronic incubation of hippocampal neurons with the GABA receptor antagonist bicuculine (BIC) that induces homeostatic down-scaling. Employing an elegant reciprocal charge reversal strategy, a CaM R126E mutant that restored normal binding affinity for PSD-95 E17R in vitro, also rescued BIC-induced PSD-95 de-clustering and GluA1 surface reduction in neurons. Thus, these findings strongly implicate Ca2+-CaM binding to PSD-95 in mediating the decreases in PSD-95 synaptic clustering and AMPAR surface expression seen following BIC treatment and suggest that increased phosphorylation of PSD-95 on T19 may promote this interaction.

Overall, up to this point the data are very clear and convincing and support the above mentioned model proposed by the authors. However, major weaknesses are that the authors fail to provide any data demonstrating that BIC treatment actually increases PSD-95-T19 phophorylation or that the PSD-95 mutations that impair CaM binding, PSD-95 de-clustering, and GluA1 surface decreases (i.e. E17R and T17K) actually impair removal of AMPARs from synapses and prevent down-scaling of synaptic strength as determined by electrophysiological recording (see below for more detail on these two issues). Until recently, it was thought that the signaling mechanisms underlying LTP/LTD and up-scaling/down-scaling must be distinct. Thus, with the key additional supporting data mentioned above, this study could make an important addition to a growing body of literature showing that shared synaptic regulatory mechanisms (in this case PSD-95-T19-P and Ca2+-CaM binding)may underlie both local, input-specific and global, homeostatic forms of synaptic plasticity (in this case both LTD and down-scaling).

Specific major comment details:

1. In order to more clearly link T19-P to homeostatic changes in PSD-95 synaptic localization in response to chronic activity elevation, the authors should use phospho-antibodies to PSD-95-T19 to examine whether the chronic BIC treatment conditions used are leading to increased phsophorylation of this site and then determine the time course of this phosphorylation response compared to the time courses of PSD-95 de-clustering and depalmitoylation (as shown in Fig.5) during chronic BIC treatment.

2. While decreased overall AMPAR surface expression is known to accompany decreased AMPAR synaptic localization and activity during homeostatic down-scaling, decreases in overall AMPAR surface expression or lack thereof alone are insufficient to conclude whether homeostatic plasticity of the synaptic AMPAR response is normal or disrupted, respectively. To support the conclusion that PSD-95 E17R and T19K suppress homeostatic down-scaling and that CaM126E rescue the impacts of E17K, the authors need to perform whole-cell voltage-clamp recording of mEPSCs to evaluate basal and BIC-induced changes in mEPSC amplitude means and cumulative distributions for each of these different conditions. Without such key supporting mEPSC data, the overall conclusion (and title of the paper) stating that Ca2+-CaM binding to PSD-95 mediates homeostatic synaptic scaling down is not adequately supported.

Referee #2:

PSD-95 is the major postsynaptic scaffold molecule at excitatory synaptic site, and regulates synaptic structure and function through the interaction with numerous proteins, including kinases (e.g. CaMKIIs, etc.), scaffold molecules (e.g. GKAP, TARPs, etc.) and glutamate receptors (AMPARs, NMDARs, etc.). PSD-95 receives many post-translational modifications (PTMs), including palmitoylation and phosphorylation, and these PTMs determine the subcellular localization of PSD-95 which are essential for its function in synaptic transmission and plasticity. However, the effect of PTMs on PSD-95 functions are still need to be addressed. The authors' group is the leader of the structural and functional studies on PSD-95 at excitatory synapses. They have previously identified that calcium/calmodulin complex (Ca2+/CaM) binds to the N-terminal 16 amino acid residues of PSD-95 and its binding is regulated by the palmitoylation of two cysteine in PSD-95. In addition, they have addressed the N-terminal structure of PSD-95, and identified that Tyr12 is important for Ca2+/CaM binding.

In this manuscript, the authors have made further efforts on elucidating the role of interaction between PSD-95 and Ca2+/CaM by fluorescence polarization assays and NMR. They also addressed the protein interaction on homeostatic plasticity by immunocytochemistry. Thr-19 (T19) of PSD-95 has been reported as the phosphorylation site of Cdk5 and GSK-3beta. The authors identified that the PSD-95 phosphorylation at T19 enhanced its binding to Ca2+/CaM. In addition, they showed that the level of palmitoylation in PSD-95 is decreased by bicuculline treatment, the typical procedure to induce homeostatic down-scaling. Immunocytochemical experiments suggest the importance of the protein interaction on homeostatic down-scaling.

The quality of structural analysis is convincingly high, but the number of concerns in the functional studies described below limit the novelty of this manuscript. In addition, T19 phosphorylation has been already established by two groups, M. Sheng and L-H. Tsai, and Dr. Sheng has reported that this phosphorylation promotes PSD-95 mobilization during LTD which shares the similar molecular mechanism with homeostatic down-scaling. Therefore, significant improvement is required to prove the physiological relevance of two protein interaction on homeostatic down-scaling. The live imaging and/or electrophysiological approaches will be necessary.

Major comments:
1. To address the role of interaction between PSD-95 and Ca2+/CaM in plasticity, the authors co-expressed shRNA against PSD-95 (shPSD-95) with shRNA-resistant PSD-95 mutants that lack the binding with CaM. (Fig. 6). They concluded that the lack of this interaction failed to induce homeostatic down-scaling evaluated by surface GluA1 staining. However, this conclusion is highly questionable as the experimental condition was not well controlled and the analysis was not convincingly performed. They have ignored the difference of synaptic targeting of exogenous PSD-95 constructs. It is clear from their result that the majority of surface GluA1s are NOT co-localized with PSD-95-WT under control and +bicuculline conditions (see Fig. 6 top). This indicates that most of GluA1 are not coupled with PSD-95 which is atypical from endogenous synaptic composition. In contrast, the PSD-95 mutants seem to have much higher expression compared with WT-replacement, and these are more specifically translocated to synaptic sites. Since the expression profile of PSD-95 constructs are very different, the current data set is not sufficient to lead the authors' conclusion. The authors should demonstrate how GluAs are removed from the PSD-95-positive postsynaptic membrane. The live imaging data will be one of the most straightforward approaches to test their hypothesis.
This concern is more evident in Fig. 7. The size of PSD-95 (WT) puncta are obviously smaller than that of two other PSD-95 mutants. This indicates that PSD-95 mutants are more specifically translocated to synaptic sites and likely induce structural synaptic plasticity (structural LTP) in spines. It is possible that enlarged spines are more resistant from bicuculline treatment. Thus, the authors have to characterize the roles of Ca2+/CaM-PSD-95 binding on basal condition before testing plasticity. The analysis of spine dimensions (spine size, length and density) and PSD-95 puncta (size and intensity) are required.

2. ALL functional studies were based on exogenously expressed PSD-95 WT and mutants, and none of the data support the link between T19-PSD-95 phosphorylation and homeostatic synaptic plasticity. The authors should first address whether T19 of endogenous PSD-95 is phosphorylated under down-scaling paradigm by using phospho-specific antibody. Then, they should test the phospho-mimic and -dead mutant (T19E-, and T19A-PSD-95) on plasticity. If T19-phosporylation

induced Ca2+/CaM-PSD-95 binding is important for down-scaling, the expression of T19E-PSD-95 should be sufficient to induce GluAR internalization.

3. The surface staining of GluA1 is not a sufficient approach for the validation of synaptic plasticity (Fig. 6 and 8). Basically, the functional study described in this manuscript is the surface staining of GluA1 ONLY. There is no guarantee that the staining represents the synaptically localized GluA1. In addition, it is possible that other GluA subunits may behave differently. They should address more detailed morphological analysis to test the role of Ca2+/CaM-PSD-95 binding in "synaptically" localized GluRs, including GluA1 and other subunits.

4. PSD-95 indirectly binds to GluRs through TARP family proteins and has multiple PTM sites that regulate subcellular localization. The authors need to validate whether CaM-binding PSD-95 mutants do not change the other binding interactions and PTMs, especially palmitoylation status.

5. The authors should perform comprehensive study to elucidate the role of protein interaction between Ca2+/CaM and PSD-95 on homeostatic plasticity. It is important to test the interaction in homeostatic upscaling induced by tetrodotoxin.

Minor comments:
1. The thorough editing is required in the methods section. First, in the immunofluorescence section, the authors should add more detailed information to acquire confocal images (objectives, zoom factor, image pixel size, Z step size, or the number of slices to be used for maximum projection images).

2. The phosphorylation of T19 of PSD-95 has described initially by L-H. Tsai's group (PMID:14749431). The authors should cite this paper.

3. As described in the Major comment 1, the quantification of the co-localization of GluA1 and exogenous PSD-95 is required. The authors should present superimposed images of PSD-95 and GluA1 channels in Fig. 6A and Fig. 8A. The correspondence of low and high magnification images in Fig 6A and 8A are not clearly shown.

4. The authors should validate the knockdown efficiency of shPSD-95 by themselves.

5. Figure 6-8: Number of rats and independent cultures are required to describe. The authors wrote in the legend, "Three dendritic segments from each of 31-42 neurons were analyzed per condition.". More detailed procedure should be described in the methods section. How these "three dendritic segments" from each neuron were chosen? Are these primary dendrite or secondary dendrite? What is the criteria of selecting dendrite? What is the total length of dendritic segments per neuron?

Referee #3:

This is a very interesting report that describes the importance of phosphorylation of PSD-95 in homeostatic down scaling. In particular, the effect of phosphorylation is on the binding with calmodulin, mediated through an electrostatic interaction. This is convincingly shown by NMR and, perhaps less convincingly, by fluorescence polarization (FP). The electrostatic interactions are confirmed by charge reversal. Interestingly, the mutations made to demonstrate effects on PSD-95/calmodulin interactions have the predicted phenotypes in cell-based experiments.

I have a few issues with the presentation:
1) The NMR experiments should be described in a bit more detail. I could not even find the field strength or the model of spectrometer. Chemical shift perturbation should be defined. Is it a perturbation of the proton, nitrogen, or a combination of both. If proton or nitrogen, why is the other ignored? If a combination, describe how that is determined.
2) The presentation of the FP experiments is worrisome. Figure 1 and Figure 4 show lines through the data, but it is not clear what these lines represent, if anything. Almost certainly, they are not one component fits to the data, both from the shape of the curves and the correspondence of the data to the dissociation constants reported in Table 1. The authors need to make clear how the data were fit (give the equations and all of the fitted parameters and error estimates) and then show the curves

based on the equations and the fitted parameters. Whenever the fitted parameters are reported, they should be reported with standard deviation or standard error.

Additional Correspondence   11 March 2017

We were able to establish homeostatic synaptic down scaling via mEPSC analysis. Accordingly we now see a clear decrease in mEPSC amplitude upon 48 h bicuculline treatment when we express WT PSD-95 and WT CaM. This decrease is gone for PSD-95 E17R and rescued by co-expression of CaM R126E in our powerful charge inversion experiment (see attached table; please note we think t-test is the correct test here between each pair of +/- BIC but the differences are also statistically significant when analyzed by ANOVA).

This finding should address the main concern of Reviewer 1 (who specifically requested exactly this mEPSC analysis) and Reviewer 2, who requested a more detailed analysis of postsynaptic AMPAR localization by IF microscopy; however, Reviewer 2 also briefly mentions electrophysiology as alternative at the end of her/his introductory write up. As mEPSC analysis is actually the only truly reliable way to determine scaling, which became only recently clear by a paper in January 2017 (Neuron 93, 646-660), I would expect that Reviewer 2 also will appreciate our recent work.

Reviewer 3 was mostly concerned with the way we analyzed our peptide titration and in fact was correct that we did make a mistake in the curve fitting. The new fittings actually result in even better values (in terms of supporting our conclusions).

We also addressed most of the other concerns.

With all that I was hoping that you would be open to a revision or new submission of our work. If you prefer I can send you a formal letter with point by point responses to all the Concerns by the Reviewers.

Thank you very much for considering my request.

Additional Correspondence   22 March 2017

Thanks for sending me the point-by-point response. I have now had a chance to take a look at everything and I do appreciate the added experiments and I think that you did a good job in addressing the referees' comments.

I would like to consider the manuscript again and I will send it back to the original referees. As you know, I do require strong support from the referees to move forward with a manuscript, which is always difficult to fully anticipate.

1st Revision - authors' response   15 April 2017

Referee #1:

This study by Turner et al. presents some very interesting new findings regarding the mechanisms through which chronic increases in neuronal activity that induce homeostatic synaptic plasticity to globally decrease excitatory synaptic strength (i.e. down-scaling)controls the subcellular localizations of the key postsynaptic density (PSD) scaffolding molecule, PSD-95, and associated AMPA-type glutamate receptors (AMPARs) that it regulates. This work builds on previous structural and functional studies from the same group of investigators showing that Ca2+-calmodulin (CaM) binds to the N-terminus of PSD-95 to inhibit its synaptic targeting by preventing palmitoylation/re-palmitoylation of Cys3 and Cys5, modifications which are essential for PSD-95 membrane and PSD targeting (Zhang et al., 2014 EMBO J). Here the authors go on to show that phosphorylation of T19 in this PSD-95 N-terminal domain increases the affinity of Ca2+-CaM binding to PSD-95. This T19-P modification was previously shown by Morgan Sheng and

colleagues to be required during the induction of long-term depression (LTD), another form of plasticity that decreases excitatory synaptic strength by promoting PSD-95 and AMPAR removal from synapses (Nelson et al., 2013 J Neurosci).

Importantly, through using NMR to determine the structure of Ca2+-CaM bound to PSD-95pT19, Turner et al. were able to identify novel electrostatic interactions between residues in CaM and PSD-95 that contribute to increased Ca2+-CaM binding affinity compared to non-phosphorylated PSD-95, including interaction of PSD-95 E17 with CaM R126. Charge reversal mutation of these key residues (including E17R and T17L in PSD-95) not only decreased the affinity of Ca2+-CaM binding to PSD-95 N-terminus in vitro but also prevented de-clustering of PSD-95 and reduction in plasma membrane surface expression of the AMPAR GluA1 subunit in response chronic incubation of hippocampal neurons with the GABA receptor antagonist bicuculine (BIC) that induces homeostatic down-scaling. Employing an elegant reciprocal charge reversal strategy, a CaM R126E mutant that restored normal binding affinity for PSD-95 E17R in vitro, also rescued BIC-induced PSD-95 de-clustering and GluA1 surface reduction in neurons. Thus, these findings strongly implicate Ca2+-CaM binding to PSD-95 in mediating the decreases in PSD-95 synaptic clustering and AMPAR surface expression seen following BIC treatment and suggest that increased phosphorylation of PSD-95 on T19 may promote this interaction.

Overall, up to this point the data are very clear and convincing and support the above mentioned model proposed by the authors. However, major weaknesses are that the authors fail to provide any data demonstrating that BIC treatment actually increases PSD-95-T19 phosphorylation or that the PSD-95 mutations that impair CaM binding, PSD-95 de-clustering, and GluA1 surface decreases (i.e. E17R and T17K) actually impair removal of AMPARs from synapses and prevent down-scaling of synaptic strength as determined by electrophysiological recording (see below for more detail on these two issues). Until recently, it was thought that the signaling mechanisms underlying LTP/LTD and up-scaling/down-scaling must be distinct. Thus, with the key additional supporting data mentioned above, this study could make an important addition to a growing body of literature showing that shared synaptic regulatory mechanisms (in this case PSD-95-T19-P and Ca2+-CaM binding) may underlie both local, input-specific and global, homeostatic forms of synaptic plasticity (in this case both LTD and down-scaling).

Specific major comment details:

1. In order to more clearly link T19-P to homeostatic changes in PSD-95 synaptic localization in response to chronic activity elevation, the authors should use phospho-antibodies to PSD-95-T19 to examine whether the chronic BIC treatment conditions used are leading to increased phosphorylation of this site and then determine the time course of this phosphorylation response compared to the time courses of PSD-95 de-clustering and depalmitoylation (as shown in Fig.5) during chronic BIC treatment.

We tried to detect an increase in T19 phosphorylation over the time course of BIC treatment (5 & 30 min, 1, 2, 24, & 48 h) by two approaches (biochemically by immunoblotting and by immunostaining with the mono-specific anti-phosphoT19 antibody) but never saw a consistent increase at any time point. The finding that our T19K mutant PSD-95 impairs both, binding of Ca/CaM and the BIC-induced reduction in surface GluA1 expression, is consistent with all of our other findings that indicate that binding of Ca/CaM to PSD-95 is critical for the downscaling. However, this finding does not show that phosphorylation of this site is really required for downscaling, as implied in Major Comment 2 by Reviewer 2. This finding hints at divergence in the mechanisms regulating local (LTD) and global forms (homeostatic downscaling) of synaptic plasticity.

We also tried to prevent BIC-induced downscaling with two different GSK3beta inhibitors (CHIR99021 and SB216763). However, the inhibitors alone caused a decrease in postsynaptic GluA1 content. After we finished our experiments with the GSK3beta inhibitors we realized that others also observed a negative effect of the GSK3beta inhibitor SB216763 and of knock down of GSK3beta on synaptic strength (Wei, Liu, and Yan, 2010: JBC 285, 26369-26376). Accordingly, GSK3beta activity is required under basal conditions for maintenance of normal postsynaptic AMPAR activity presumably by acting via mechanisms that are different from those responsible for LTD as reported by M. Sheng and others. This requirement prevented us from further exploring the

role of GSK3beta in homeostatic synaptic down scaling. These unpublished data are briefly described in the Discussion.

Given these limitations, we no longer claim a role of T19 phosphorylation in scaling down. **Still, the pT19_PSD-95 peptide proved useful for deducing the structure of the Ca/CaM-PSD-95 complex and identifying the salt bridge formed between E17 in PSD-95 and R126 in CaM, which enabled the potent charge inversion experiments.**

2. While decreased overall AMPAR surface expression is known to accompany decreased AMPAR synaptic localization and activity during homeostatic down-scaling, decreases in overall AMPAR surface expression or lack thereof alone are insufficient to conclude whether homeostatic plasticity of the synaptic AMPAR response is normal or disrupted, respectively. To support the conclusion that PSD-95 E17R and T19K suppress homeostatic down-scaling and that CaM126E rescue the impacts of E17K, the authors need to perform whole-cell voltage-clamp recording of mEPSCs to evaluate basal and BIC-induced changes in mEPSC amplitude means and cumulative distributions for each of these different conditions. Without such key supporting mEPSC data, the overall conclusion (and title of the paper) stating that Ca2+-CaM binding to PSD-95 mediates homeostatic synaptic scaling down is not adequately supported.

We now provide a full extensive set of mEPSC recordings that clearly show that mEPSC amplitude is decreased over the whole synapse population by BIC treatment, that **this decrease is prevented when E17 is mutated to Arg, and that co-expression of CaMR126E rescues the downscaling**. Such charge inversion experiments are in fact the only way to unequivocally show that a specific protein interaction mediates a certain effect. Our new mESPC experiments now prove that Ca/CaM binding to PSD-95 is required for homeostatic synaptic down scaling.

Referee #2:

PSD-95 is the major postsynaptic scaffold molecule at excitatory synaptic site, and regulates synaptic structure and function through the interaction with numerous proteins, including kinases (e.g. CaMKIIs, etc.), scaffold molecules (e.g. GKAP, TARPs, etc.) and glutamate receptors (AMPARs, NMDARs, etc.). PSD-95 receives many post-translational modifications (PTMs), including palmitoylation and phosphorylation, and these PTMs determine the subcellular localization of PSD-95 which are essential for its function in synaptic transmission and plasticity. However, the effect of PTMs on PSD-95 functions are still need to be addressed. The authors' group is the leader of the structural and functional studies on PSD-95 at excitatory synapses. They have previously identified that calcium/calmodulin complex (Ca2+/CaM) binds to the N-terminal 16 amino acid residues of PSD-95 and its binding is regulated by the palmitoylation of two cysteine in PSD-95. In addition, they have addressed the N-terminal structure of PSD-95, and identified that Tyr12 is important for Ca2+/CaM binding.
In this manuscript, the authors have made further efforts on elucidating the role of interaction between PSD-95 and Ca2+/CaM by fluorescence polarization assays and NMR. They also addressed the protein interaction on homeostatic plasticity by immunocytochemistry. Thr-19 (T19) of PSD-95 has been reported as the phosphorylation site of Cdk5 and GSK-3beta. The authors identified that the PSD-95 phosphorylation at T19 enhanced its binding to Ca2+/CaM. In addition, they showed that the level of palmitoylation in PSD-95 is decreased by bicuculline treatment, the typical procedure to induce homeostatic down-scaling. Immunocytochemical experiments suggest the importance of the protein interaction on homeostatic down-scaling.
The quality of structural analysis is convincingly high, but the number of concerns in the functional studies described below limit the novelty of this manuscript. In addition, T19 phosphorylation has been already established by two groups, M. Sheng and L-H. Tsai, and Dr. Sheng has reported that this phosphorylation promotes PSD-95 mobilization during LTD which shares the similar molecular mechanism with homeostatic down-scaling. Therefore, significant improvement is required to prove the physiological relevance of two protein interaction on homeostatic down-scaling. The live imaging and/or electrophysiological approaches will be necessary.

Major comments:

1. To address the role of interaction between PSD-95 and Ca2+/CaM in plasticity, the authors co-expressed shRNA against PSD-95 (shPSD-95) with shRNA-resistant PSD-95 mutants that lack the binding with CaM (Fig. 6). They concluded that the lack of this interaction failed to induce homeostatic down-scaling evaluated by surface GluA1 staining. However, this conclusion is highly questionable as the experimental condition was not well controlled and the analysis was not convincingly performed. They have ignored the difference of synaptic targeting of exogenous PSD-95 constructs. It is clear from their result that the majority of surface GluA1s are NOT co-localized with PSD-95-WT under control and +bicuculline conditions (see Fig. 6 top). This indicates that most of GluA1 are not coupled with PSD-95 which is atypical from endogenous synaptic composition. In contrast, the PSD-95 mutants seem to have much higher expression compared with WT-replacement, and these are more specifically translocated to synaptic sites. Since the expression profile of PSD-95 constructs are very different, the current data set is not sufficient to lead the authors' conclusion. The authors should demonstrate how GluAs are removed from the PSD-95-positive postsynaptic membrane. The live imaging data will be one of the most straightforward approaches to test their hypothesis.
This concern is more evident in Fig. 7. The size of PSD-95 (WT) puncta are obviously smaller than that of two other PSD-95 mutants. This indicates that PSD-95 mutants are more specifically translocated to synaptic sites and likely induce structural synaptic plasticity (structural LTP) in spines. It is possible that enlarged spines are more resistant from bicuculline treatment. Thus, the authors have to characterize the roles of Ca2+/CaM-PSD-95 binding on basal condition before testing plasticity. The analysis of spine dimensions (spine size, length and density) and PSD-95 puncta (size and intensity) are required.

We appreciate these critical, detailed-oriented and especially helpful comments. Please note that the GluA1 signals were generally stronger than the GFP-PSD-95 signals perhaps because the GluA1 signals were amplified by primary and secondary antibody staining while PSD-95 signal arose from the relatively weak GFP fluorescence. Accordingly, a number of GluA1 puncta might not show GFP-PSD-95 signals due to lack of sensitivity rather than lack of presence of GFP-PSD-95. We would submit that most of the stronger sGluA1 signals were also positive for GFP-PSD-95. However, we now realize that the original sample image for the WT PSD-95 control condition was not optimally chosen. We now show a better representation of our images seen with WT PSD-95, which exhibits much more extensive co-labeling with sGluA1 puncta than the original images in Fig. 6 (now Fig. 5). We also provide now red-green overlay images for GluA1 and PSD-95 in current Fig. 5 & 7. As rigorous read-out we now provide quantification of the degree of overlap between GFP-PSD-95 and sGluA1 signal in Fig. EV 5. Importantly, the degree of co-localization of GFP-PSD-95 and sGluA1 signals is not different for PSD-95 WT versus E17R and T19K.

Similarly, we understand now that we did not optimally select the sample image for WT GFP-PSD-95 under control conditions in Fig. 7 (now Fig. 6) with regard to puncta size and intensity. The images were meant to represent enrichment within spine head versus dendritic shaft. Importantly, spine enrichment of WT GFP-PSD-95 and the mutants are similar under basal conditions (Fig. 7B, now Fig. 6D) supporting comparable synaptic targeting. Nevertheless, we replaced the sample image for WT GFP-PSD-95 for control condition with a more representative image showing PSD-95 puncta comparable to the other panels. Given the different levels of ectopic expression from neuron to neuron, we believe that spine enrichment measurement, which provides correction for such differences, is more suitable to assess synaptic localization than absolute puncta intensity. However, we performed new extensive analysis of PSD-95 puncta size and intensity for all GFP-PSD-95 variants under basal conditions as well as spine head size, length and density (Fig. 6E-I). None of these parameters are significantly different for PSD-95 WT versus E17R and T19K. These data show that the PSD-95 mutants are synaptic targeted to a degree that is similar to WT and do not alter the spines under basal conditions. Moreover, a lack of effect by the E17R mutation on basal synaptic function is supported by the mEPSC analysis (new Fig. 8); accordingly, mEPSC amplitudes, frequencies, and decay time constants are comparable for PSD-95 WT and E17R under basal conditions.

2. ALL functional studies were based on exogenously expressed PSD-95 WT and mutants, and none of the data support the link between T19-PSD-95 phosphorylation and homeostatic synaptic plasticity. The authors should first address whether T19 of endogenous PSD-95 is phosphorylated under down-scaling paradigm by using phospho-specific antibody. Then, they should test the phospho-mimic and -dead mutant (T19E-, and T19A-PSD-95) on plasticity. If T19-phosporylation

induced Ca2+/CaM-PSD-95 binding is important for down-scaling, the expression of T19E-PSD-95 should be sufficient to induce GluAR internalization.

We tried to detect the hypothesized increase in pT19 over a time course of BIC treatment but were not able to do so as discussed above (please see response to Referee 1 Concern # 21).

The T19A mutant would be expected to result in an effect that is more subtle than the charge inversion T19K mutation. Because T19K did not alter PSD-95 spine enrichment nor surface expression of GluA1, we did not pursue the T19A mutation.

Because T19 phosphorylation was not detected upon BIC treatment, we did not pursue the analysis of phospho-mimic and -dead mutants for their effects on downscaling. Also, the T19E mutation has a rather modest effect on the affinity of Ca/CaM binding (it decrease the Kd value from 22.5 to 18.5 microM when the phosphoT19 decreases the Kd to 8.6 microM; please see Table 1). Such a modest effect of the T19E mutation compared to the actual phosphorylation is not surprising because this mutation does not necessarily mimic phosphorylation well enough with the phosphate group being much larger and also more negatively charged than the glu side chain. Thus we would not expect a detectable effect especially as the BIC-induced down scaling is very modest in magnitude (~15% decrease in mEPSC amplitude, as typically reported by others).

3. The surface staining of GluA1 is not a sufficient approach for the validation of synaptic plasticity (Fig. 6 and 8). Basically, the functional study described in this manuscript is the surface staining of GluA1 ONLY. There is no guarantee that the staining represents the synaptically localized GluA1. In addition, it is possible that other GluA subunits may behave differently. They should address more detailed morphological analysis to test the role of Ca2+/CaM-PSD-95 binding in "synaptically" localized GluRs, including GluA1 and other subunits.

As Reviewer 1 points out, determining whether homeostatic scaling has occurred is best accomplished by mEPSC analysis. In fact, new work now indicates that recruiting AMPARs to postsynaptic sites does not necessarily translate into a stronger postsynaptic response to presynaptic glutamate release as determined by mEPSC analysis (Sinnen et al., 2017: Neuron 93, 646-660). Thus we decided to focus our efforts to perform mEPSC analysis, which now clearly shows the relevance of Ca/CaM binding to PSD95 in BIC-induced down scaling (please see response to Referee 1 Concern # 2).

4. PSD-95 indirectly binds to GluRs through TARP family proteins and has multiple PTM sites that regulate subcellular localization. The authors need to validate whether CaM-binding PSD-95 mutants do not change the other binding interactions and PTMs, especially palmitoylation status.

We showed earlier that the Y12E mutation of PSD-95 does not affect its palmitoylation under basal conditions (please see Figure 5A,B in Zhang et al., 2014: EMBO J 33, 1341-1353). Given that E17 and T19 are farther away than Y12 from the palmitoylation site at the very N-terminus of PSD-95 (Cys 3 and Cys 5), we did not expect any changes in palmitoylation for E17R and T19K either. However, we now provide evidence that this is indeed the case (Fig. EV3). We further show that co-immunoprecipitation of AMPARs and NMDARs with PSD-95 is not affected by either mutation (Fig. EV4).

5. The authors should perform comprehensive study to elucidate the role of protein interaction between Ca2+/CaM and PSD-95 on homeostatic plasticity. It is important to test the interaction in homeostatic upscaling induced by tetrodotoxin.

On a molecular level homeostatic synaptic upscaling is not plainly the reversal of downscaling. Tetrodotoxin would decrease basal activity of glutamatergic synapses, further decreasing the low resting Ca concentration inside spines and further decrease the interaction of Ca/CaM with PSD-95, which is already minimal under basal conditions. Thus we do not expect that this interaction plays a role in homeostatic synaptic upscaling during chronic blockade of activity.

Minor comments:
1. The thorough editing is required in the methods section. First, in the immunofluorescence section, the authors should add more detailed information to acquire confocal images (objectives, zoom

factor, image pixel size, Z step size, or the number of slices to be used for maximum projection images).
This information is now provided in the Methods section under "Immunofluorescence".

2. The phosphorylation of T19 of PSD-95 has described initially by L-H. Tsai's group (PMID:14749431). The authors should cite this paper.
We now cite this publication. Please note that the authors conclude that in vitro both T19 and S25 can be phosphorylated by cdk5 but S25 seems to be the primary target in vitro. The authors did show with a phospho-specific antibody against pS25 that this site is indeed phosphorylated in vivo but did not provide analogous data for pT19. I recently e-mailed Dr. Tsai and she responded that she never determined with certainty that T19 is phosphorylated by cdk5 in vivo or in intact neurons.

3. As described in the Major comment 1, the quantification of the co-localization of GluA1 and exogenous PSD-95 is required. The authors should present superimposed images of PSD-95 and GluA1 channels in Fig. 6A and Fig. 8A. The correspondence of low and high magnification images in Fig 6A and 8A are not clearly shown.
We now provide quantification of the degree of colocalization between GFP-PSD-95 and sGluA1 signal in Fig. EV 5. Accordingly, the degree of co-localization of GFP-PSD-95 and sGluA1 signals does not differ between PSD-95 WT, E17R, and T19K. We now show superimposed images in original Fig. 6 and 8 (current Fig. 5 and 7) and label the segments in the low magnifications that are shown in high magnifications.

4. The authors should validate the knockdown efficiency of shPSD-95 by themselves.
We now show that our molecular replacement plasmid nearly completely eliminates endogenous PSD-95 migrating just below 100 kDa and that the ectopically expressed GFP-tagged PSD-95 migrating ~ 130 kDa is similar in expression levels as endogenous PSD-95 when not knocked down, as determined by immunoblotting with an antibody against PSD-95 that recognizes endogenous PSD-95 and GFP-tagged PSD-95 (Fig. EV2).

5. Figure 6-8: Number of rats and independent cultures are required to describe. The authors wrote in the legend, "Three dendritic segments from each of 31-42 neurons were analyzed per condition.". More detailed procedure should be described in the methods section. How these "three dendritic segments" from each neuron were chosen? Are these primary dendrite or secondary dendrite? What is the criteria of selecting dendrite? What is the total length of dendritic segments per neuron?
This information is now provided in the Methods section under "Immunofluorescence".

Referee #3:

This is a very interesting report that describes the importance of phosphorylation of PSD-95 in homeostatic down scaling. In particular, the effect of phosphorylation is on the binding with calmodulin, mediated through an electrostatic interaction. This is convincingly shown by NMR and, perhaps less convincingly, by fluorescence polarization (FP). The electrostatic interactions are confirmed by charge reversal. Interestingly, the mutations made to demonstrate effects on PSD-95/calmodulin interactions have the predicted phenotypes in cell-based experiments.

I have a few issues with the presentation:
1) The NMR experiments should be described in a bit more detail. I could not even find the field strength or the model of spectrometer. Chemical shift perturbation should be defined. Is it a perturbation of the proton, nitrogen, or a combination of both. If proton or nitrogen, why is the other ignored? If a combination, describe how that is determined.

This information is now provided in great detail in the Methods section under "NMR Spectroscopy." The Chemical shift perturbation is now defined in the legend to Fig. 1B.

2) The presentation of the FP experiments is worrisome. Figure 1 and Figure 4 show lines through the data, but it is not clear what these lines represent, if anything. Almost certainly, they are not one component fits to the data, both from the shape of the curves and the correspondence of the data to the dissociation constants reported in Table 1. The authors need to make clear how the data were fit (give the equations and all of the fitted parameters and error estimates) and then show the curves

based on the equations and the fitted parameters. Whenever the fitted parameters are reported, they should be reported with standard deviation or standard error.

We appreciate this comment, making us aware of an error in our calculations. The dissociation constant (K_D) for each titration is now calculated according to a one-site binding model:

$$\text{Polarization (\% change)} = B_{max} * \left(\frac{[\text{Calmodulin}]_{free}}{[\text{Calmodulin}]_{free} + K_D} \right)$$

where B_{max} is a normalization scaling factor (normalized for the polarization change at saturation) and K_D is the calculated dissociation constant.

The FP data in Figs. 3 and EV1 are now properly fitted to a one-site model and the calculated dissociation constants are listed in Table 1.

2nd Editorial Decision   19 May 2017

Thanks for sending us your revised manuscript. The original two referees have now reviewed your study and their comments are provided below.

Both referees appreciate that the analysis has been strengthened and referee #1 is supportive of the manuscript as is. Referee #2 has some good remaining issues - see referee comments below - and resolving those would add a lot to the manuscript also in light of that the role of the PSD-95 T16 phosphorylation is no longer linked to homeotic scaling.

I would therefore like to invite you to submit a suitably revised manuscript that addresses the concerns raised by referee #2.

Let me know if we need to discuss anything further

REFEREE REPORTS

Referee #1:

The authors have done an excellent job of responding to criticisms raised in the previous reviews. In particular the addition of the mEPSC recording data greatly strengthens the manuscript and increase impact. I recommend publication in EMBO J with only minor revisions (see below)

Minor points:

1. Page 4 Introduction: Kim and Ziff, 2014 only showed that decreased calcineurin/PP2B phosphatase activity favors GluA1 S845 phosphorylation by PKA during TTX-induced scaling up. They did not specifically address the role of AKAP5 anchoring of calcineurin/PP2B in this study, thus this AKAP5-PP2B mechanism remains to be explored. The wording of this reference to Kim and Ziff, 2014 should be changed accordingly to more accurately reflect the findings of that study.

2. In this same section of the introduction, for the sake of completeness the authors may also want consider citing Siddoway et al., 2013 J. Neurosci that found a role for PP1 phosphatase activity and regulation of GluA2 S880 phosphorylation in AMPAR removal from synapses in homeostatic downscaling.

Referee #2:

In the revised manuscript, the authors dropped the context on the functional significance of PSD-95 phosphorylation in homeostatic synaptic plasticity, the very important/attractive hypothesis explaining the molecular mechanism important for the plasticity. Although newly added structural and functional data indicates the importance of Ca/CaM and PSD-95 binding in down-scaling, this manuscript lacks the translocation mechanism of PSD-95 from membrane (achieved by palmitoylation of PSD-95) to intracellular (by Ca/CaM binding to PSD-95) domain. Therefore, they should perform further experiments/analysis to present the concreate evidence to

highlight the importance of the balance between palmitoylation and Ca/CaM-binding status of PSD-95 on AMPAR surface expression, spine morphology and synaptic function.

Major comments:
#1: The authors stated in the rebuttal comments that the phosphorylation of T19 site of PSD-95 was not detected by the induction of homeostatic down-scaling, ruling out the possibility of phosphorylation-dependent dispersal of PSD-95 from synapses. As the lack of T19 phosphorylation highlights the difference from the model of PSD-95 in LTD (Nelson et al., 2013), their negative result is very important for the neuroscience society. The authors should mention and discuss their finding.
Instead of addressing the functional significance of PSD-95 T19 phosphorylation, the authors focused on the structural and functional analysis of Ca/CaM-PSD-95 binding in plasticity by using CaMR126E and PSD-95E17R mutants. They have nicely shown that CaM-unbound PSD-95 mutant, PSD-95E17R, failed to induce plasticity, but this mutant gained its function when co-existed with CaM mutant R126E, the charge inversion mutant enabled to bind to PSD-95 mutant. This is a very interesting observation and the authors should show this gain-of-function result is inversely correlated with the level of PSD-95 palmitoylation. The authors should test whether the status of palmitoylation of exogenous PSD-95 mutant, including E17R and wild-type control, are regulated by bicuculline and CaM mutant (R126E and wild-type control) in neuronal cultures, the similar approach used in Fig. EV3. This will be very important control experiments to confirm the authors' hypothesis proposed in the present and previous reports (Zhang, et al., 2014).

#2 The authors have not fully addressed the point which I have raised in the major comment #1. Activity-dependent synaptic localization of PSD-95 mutants and synaptic structure is the most important analysis the authors should perform. They could easily compare the structural differences with or without bicuculline treatment in the neurons infected with different PSD-95 mutants, since they already presented the effect of PSD-95 mutants on "basal" status in Fig. 6D-I. As all the images required are already in their hands, the analysis of spine dimension should be carried out immediately.

2nd Revision - authors' response   07 September 2017

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We now state more precisely: "*In the context of scaling up, S845 phosphorylation is further augmented by decreased dephosphorylation by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin/PP2B as the reduction in neuronal activity that leads to scaling up decreases Ca^{2+} influx (Kim and Ziff, 2014).*"

2. In this same section of the introduction, for the sake of completeness the authors may also want consider citing Siddoway et al., 2013 J. Neurosci that found a role for PP1 phosphatase activity and regulation of GluA2 S880 phosphorylation in AMPAR removal from synapses in homeostatic downscaling.

We now state (**new text in bold**): "*The reversal of S845 phosphorylation appears to contribute to scaling down (Diering et al., 2014) as does ubiquitination and proteasomal degradation of AMPARs (Hou et al., 2011)* **and disinhibition (i.e., activation) of the phosphatase PP1 due to**"

phosphorylation of its endogenous antagonist inhibitor-2 by the Ca^{2+}/calmodulin-dependent myosin light chain kinase, which might contribute to scaling down by dephosphorylating the AMPAR GluA2 subunit on S880 (Siddoway et al., 2013)."

Referee #2:

In the revised manuscript, the authors dropped the context on the functional significance of PSD-95 phosphorylation in homeostatic synaptic plasticity, the very important/attractive hypothesis explaining the molecular mechanism important for the plasticity. Although newly added structural and functional data indicates the importance of Ca/CaM and PSD-95 binding in down-scaling, this manuscript lacks the translocation mechanism of PSD-95 from membrane (achieved by palmitoylation of PSD-95) to intracellular (by Ca/CaM binding to PSD-95) domain.
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We performed further analysis of T19 phosphorylation over the course of bicuculline treatment to further confirm and be as certain as possible our original findings that there is apparently no detectable increase in T19 phosphorylation (new Figure EV5). Although immunoblots are very clean and well controlled and our data are very solid, they are negative and it is impossible to exclude a minor decrease in T19 phosphorylation or loss of phosphoT19 during sample preparation, which could result in equalizing phosphoT19 levels (one can never be certain that all phosphatase activity is blocked during sample preparation despite the use of a well defined and potent mix of phosphatase inhibitors in our samples; please note that we also tried to use immunofluorescent staining after fixation of hippocampal cultures with the phospho-specific antibody against T19 but stainings were qualitatively not satisfactory). We cannot rule out a decrease that is too small to detect but 'chronic' (i.e., it is occurring over an expanded period during bicuculline treatment), which could augment the loss of spine PSD-95 during bicuculline treatment. Thus we are hesitant to draw any firm conclusions based on a negative finding in the *Result* section but rather only discuss more carefully the possibility that molecular mechanisms of homeostatic down scaling of synaptic strength differs from those mediating LTD.

Instead of addressing the functional significance of PSD-95 T19 phosphorylation, the authors focused on the structural and functional analysis of Ca/CaM-PSD-95 binding in plasticity by using CaMR126E and PSD-95E17R mutants. They have nicely shown that CaM-unbound PSD-95 mutant, PSD-95E17R, failed to induce plasticity, but this mutant gained its function when co-existed with CaM mutant R126E, the charge inversion mutant enabled to bind to PSD-95 mutant. This is a very interesting observation and the authors should show this gain-of-function result is inversely correlated with the level of PSD-95 palmitoylation. The authors should test whether the status of palmitoylation of exogenous PSD-95 mutant, including E17R and wild-type control, are regulated by bicuculline and CaM mutant (R126E and wild-type control) in neuronal cultures, the similar approach used in Fig. EV3. This will be very important control experiments to confirm the authors' hypothesis proposed in the present and previous reports (Zhang, et al., 2014).

Our combined structural and resulting functional analysis using charge inversion identifies binding of Ca^{2+}/CaM to the N-terminus of PSD-95 as a critical molecular event in homeostatic down scaling of synaptic strength. It provides a direct link between the increase in Ca^{2+} influx during bicuculline treatment and molecular postsynaptic changes, thereby substantially advancing our mechanistic understanding of down scaling. Based on our earlier work (Zhang et al., 2014) that shows that Ca^{2+}/CaM binding to the N-terminus antagonizes palmitoylation, we propose that Ca^{2+}/CaM acts in part by reducing palmitoylation, which is essential for postsynaptic PSD-95 localization (El-Husseini et al., 2002). In support of this hypothesis we found that bicuculline treatment resulted in a

clear decrease in PSD-95 palmitoylation (Fig. 4). We agree that it would be desirable to establish that this decrease is prevented when Ca/CaM binding is impaired by mutating PSD-95 (i.e., E17R) and that combining PSD-95 E17R with CaM R126E restores the decrease in palmitoylation. However, this analysis is much more difficult than our original analysis of palmitoylation of endogenous PSD-95 in hippocampal cultures and of WT and E17R mutant PSD-95 in HEK293 cells because such a biochemical analysis would require that the WT and mutant forms of PSD-95 and CaM are co-expressed in the majority (>80%) of the cultured neurons. Multiple trials with viral infections were not successful likely because achieving double infections of the majority of neurons with two different viruses is very difficult at best. After several fruitless trials to extensively co-express PSD-95 and CaM, we performed several experiments to establish immunostaining with an antibody that specifically recognizes palmitoylated PSD-95 as developed by Dr. Masaki Fukata and colleagues, which requires much lower dual infection levels than a biochemical analysis.

In support of the notion that activity-induced PSD-95-CaM binding mediates removal of PSD-95 and with it AMPARs at least in part by reducing PSD-95 palmitoylation we found with this analysis that palmitoylation is in fact reduced when testing PSD-95 WT (see figure below). Furthermore, this reduction is not seen for PSD-95 E17R when expressed in combination with CaM WT, again very much in support of that notion. However, combining PSD-95 E17R with CaM R126E resulted only in a partial rescue of the decrease in palmitoylation (8% reduction) right in between the 20% reduction we see with PSD-95 WT and the 0% reduction we see with PSD-95 E17R with this approach. Of note, there is no statistically significant difference when comparing bicuculline-induced changes seen for the full charge inversion condition (PSD-95 E17R / CaM R126E) with either PSD-95 WT / CaM WT or PSD-95 E17R / CaM WT. It is quite conceivable that the full charge inversion is only partially rescuing the decrease in palmitoylation because it cannot fully mimic the precise conformation of the PSD-95 – CaM complex. As these data are not an ultimate proof for the hypothesis that Ca/CaM acts here by decreasing palmitoylation we only present those data in this letter (see Figure below).
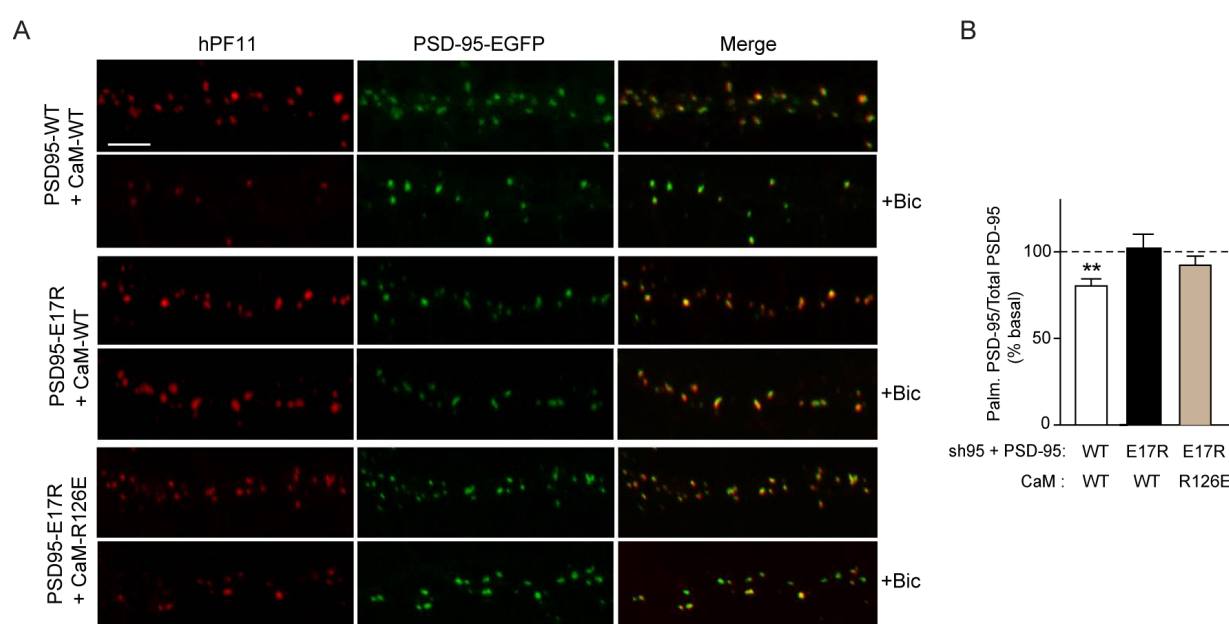

Figure: CaM R126E partially restores the bicuculline-induced decrease in PSD-95 palmitoylation, which is impaired for PSD-95 E17R.

Cultured hippocampal neurons were sequentially infected at 12 DIV with lentivirus expressing WT or R126E CaM-IRES-ECFP and at 14 DIV with lentivirus expressing shRNA against PSD-95 (sh95) and sh-resistant WT or E17R PSD-95-EGFP. If indicated, cultures were treated with bicuculline (Bic; 50 μM) at 17 DIV for 48 h before fixation/permeabilization for 10 min in cold (-20°C) methanol, washing with PBS, blocking with 1% BSA in PBS for 30 min, and incubation with hPF11 (gift from Dr. Masaki Fukata, Okazaki, Japan), a recombinant antibody specific for palmitoylated PSD-95 (Y. Fukata, A. Dimitrov, G. Boncompain, O. Vielemeyer, F. Perez, and M.

Fukata 2013: J Cell Biol 202, 145-161), in PBS containing 1% BSA for 2 h at room temperature. Following washes with PBS and blocking for 1 h, samples were incubated with AlexaFluor 647-conjugated anti-human IgG Fc-specific secondary antibody (Jackson ImmunoResearch) in PBS containing 1% BSA for 1 h. After final washes with PBS, coverslips were mounted onto glass slides using ProLong Gold AntiFade reagent (Molecular Probes). Fluorescence images of secondary and tertiary dendrites of infected neurons were acquired on an LSM700 confocal microscope (Zeiss) using a 63x oil-immersion objective (NA 1.4) and optical zoom of 1.5, as a Z-series of 8-10 slices. All conditions within the same experiment were imaged using the same microscope settings. Maximum intensity projection images were analyzed with MetaMorph Imaging (Molecular Devices). Average fluorescence intensities per square area for the total PSD-95-GFP signal as well as the hPF11 signal were determined within traced GFP-positive puncta along 50-70 μm dendritic segments. Data were averaged per neuron, and means from several neurons were then averaged to obtain a final mean for each condition. Subsequently, data from 2 different independent experiments were pooled to obtain the mean for each condition.

(A) Representative confocal microscopic images of dendritic segments showing intrinsic EGFP fluorescence (green) and hPF11 immunostaining (red; scale bar: 5 μm).

(B) Quantification of hPF11 intensity (Palm. PSD-95) normalized to GFP intensity (Total PSD-95) from (A) showed Bic-induced reduction in PSD-95 palmitoylation in neurons expressing PSD-95 WT but not in those expressing PSD-95 E17R in the presence of CaM WT. Coexpression of CaM R126E with PSD-95 E17R appeared to partially rescue the effect of BIC on PSD-95 palmitoylation. Dendritic segments from 19-20 neurons from two independent sets of cultures were analyzed per condition (**p<0.01 vs. untreated basal levels; student's t test).

#2 The authors have not fully addressed the point which I have raised in the major comment #1. Activity-dependent synaptic localization of PSD-95 mutants and synaptic structure is the most important analysis the authors should perform. They could easily compare the structural differences with or without bicuculline treatment in the neurons infected with different PSD-95 mutants, since they already presented the effect of PSD-95 mutants on "basal" status in Fig. 6D-I. As all the images required are already in their hands, the analysis of spine dimension should be carried out immediately.

With respect to our original sample images in Fig. 7 Reviewer 2 noted *previously "the size of PSD-95 (WT) puncta are obviously smaller than that of two other PSD-95 mutants. This indicates that PSD-95 mutants are more specifically translocated to synaptic sites and likely induce structural synaptic plasticity (structural LTP) in spines. It is possible that enlarged spines are more resistant from bicuculline treatment. Thus, the authors have to characterize the roles of Ca2+/CaM-PSD-95 binding on* **basal condition** *(high lighted by the respondent) before testing plasticity. The analysis of spine dimensions (spine size, length and density) and PSD-95 puncta (size and intensity) are required."* We understood that Reviewer 2 originally wanted to know whether expression of WT vs the two PSD-95 mutants affects spine size under basal conditions. Also, earlier work did not provide evidence for a change in spine size during scaling up (Soares et al., 2013) and to the best of our knowledge no analogous evidence had been available for a decrease in spine size during scaling down of mEPSC amplitude. This actually just changed with the most recent work by Schratt and co-workers published in May 2017 in *EMBO J* (Rajman et al., 2017, as now cited in our MS). Accordingly, inhibition of GABAergic synaptic transmission with picrotoxin or bicuculline led to a 15-20% reduction in the average spine size. As now requested by Reviewer 2, we now expanded our analysis of spine dimensions under control conditions to include such analysis of existing images from bicuculline-treated cultures. We observed a decrease of ~5% in spine head diameter upon bicuculline versus control treatment, which did not reach statistical significance. Notably, BIC increased spine head diameter in neurons expressing the E17R and T19K mutants by ~9% and ~3%, respectively, augmenting the actual difference in spine size changes between WT and mutants although none of these effects reached statistical significance. A small increase rather than decrease in spine size was also seen upon chronic picrotoxin treatment when miR129 was down regulated with anti-miR129 plasmid (Fig. 2 in Rajman et al., 2017) and when the miR129 targets Atp2b4 and doublecortin (DCX) were ectopically expressed (Fig. 6 in Rajman et al., 2017) to prevent their down regulation by miR129 during homeostatic synaptic down scaling, which requires miR129 expression and the consequent down regulation of Atp2b4 and DCX (Rajman et al., 2017). These results suggest that when mechanisms of down scaling are blocked the increase in network activity can

actually drive a modest increase in spine size. Notably, this modest increase in spine size doesn't seem to be translating into an increase in mEPSC amplitude either in the work by Rajman et al. 2017 (Fig. 2 and 6), or in our hands (Figure 8 of our manuscript; such dissociation between spine head size and AMPAR content has been previously reported for LTD and homeostatic scaling (Beique et al., 2011; Sdrulla and Linden, 2007; Soares et al., 2013; Wang et al., 2007)). This consideration is also consistent with our original finding that preventing Ca/CaM from binding to the N-terminus of PSD-95 not only impaired the diffusion of PSD-95 out of spines during short periods of strong Ca^{2+} influx but actually allowed an increase in spine content of PSD-95 (Zhang et al., 2014).

However, we find a clear **increase in spine neck length of 20-30% for WT and the PSD-95 mutants**. This spine neck lengthening during homeostatic synaptic down scaling is to our knowledge a novel finding. We discuss these findings now and propose that bicuculline-induced spine neck lengthening can be expected to decrease the conduction of electric signals from spine to shaft and thereby of their integration, which would contribute to a reduction of the postsynaptic response as detected at the cell soma. Given that bicuculline-induced spine neck elongation is seen upon transfection with WT as well as both PSD-95 mutants it is obviously independent of binding of Ca/CaM to the N-terminus of PSD-95 and its displacement and thus might constitute a parallel mechanism for homeostatic down scaling of postsynaptic signals reaching the soma.

Referee #3:

No further concerns were noted.

Accepted	02 October 2017

Thank you for sending us your revised manuscript. Your study has now been re-reviewed by referee # 2 and the comments are provided below. Referee #2 appreciates the introduced changes and I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORT

Referee #2:

I understood the comments in the second rebuttal letter. It is a significant advance to the field and I think it will be well-cited. In sum, I have no remaining major concerns.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Johannes W. Hell
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95829R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

→ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
→ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
→ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
→ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
→ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

→ a specification of the experimental system investigated (eg cell line, species name).
→ the assay(s) and method(s) used to carry out the reported observations and measurements
→ an explicit mention of the biological and chemical entity(ies) that are being measured.
→ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

→ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
→ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
→ a statement of how many times the experiment shown was independently replicated in the laboratory.
→ definitions of statistical methods and measures:
  • common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  • are tests one-sided or two-sided?
  • are there adjustments for multiple comparisons?
  • exact statistical test results, e.g., P values = x but not P values < x;
  • definition of 'center values' as median or average;
  • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes for our biochemical, electrophysiological and immunofluorescence analyses was chosen based on extensive previously published work indicating that sample sizes were adequate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Untreated and Bic-treated coverslips were interleaved for mEPSC recordings.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For key imaging experiments, analysis was done in blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	
Is there an estimate of variation within each group of data?	Standard error of mean (SEM) is depicted for all data.
Is the variance similar between the groups that are being statistically compared?	Variance between statistically compared groups is assumed to be simiar due to the similar sample size between groups

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See "Materials and Methods"
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293 were from standard source and tested for mycoplasma with negative results

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The NMR assignments have been deposited in BMRB (accession number 30062). The atomic coordinates have been deposited in the Protein Databank (5J7J).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA